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Impact of oilseed rape expressing the insecticidal serine protease inhibitor, mustard trypsin inhibitor-2 on the beneficial predator *Pterostichus madidus*

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Abstract

Insect-resistant transgenic plants have been suggested to have deleterious effects on beneficial predators feeding on crop pests, through transmission of the transgene product by the pest to the predator. To test this hypothesis, effects of oilseed rape expressing the serine protease inhibitor, mustard trypsin inhibitor –2 (MTI-2), on the predatory ground beetle *Pterostichus madidus* were investigated, using diamondback moth, *Plutella xylostella* as the intermediary pest species. As expected, oilseed rape expressing MTI-2 had a deleterious effect on the development and survival of the pest. However, incomplete pest mortality resulted in survivors being available to predators at the next trophic level, and inhibition studies confirmed the presence of biologically active transgene product in pest larvae. Characterization of proteolytic digestive enzymes of *P. madidus* demonstrated that adults utilize serine proteases with trypsin-like and chymotrypsin-like specificities; the former activity was completely inhibited by MTI-2 *in vitro*. When *P. madidus* consumed prey reared on MTI-2 expressing plants over the reproductive period in their life cycle, no significant effects upon survival were observed as a result of exposure to the inhibitor. However, there was a short-term significant inhibition of weight gain in female beetles fed unlimited prey containing MTI-2, with a concomitant reduction of prey consumption. Biochemical analyses showed that the inhibitory effects of MTI-2 delivered via prey on gut proteolysis in the carabid decreased with time of exposure, possibly resulting from up-regulation of inhibitor-insensitive proteases. Of ecological significance, consumption of MTI-2 dosed prey had no detrimental effects on reproductive fitness of adult *P. madidus*.

Keywords: beneficial predators, carabid, mustard trypsin inhibitor-2 (MTI-2), transgenic oilseed rape, tritrophic interactions

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Introduction

Modern biotechnology is an important new tool in the development of sustainable agricultural systems. Increasing varietal resistance based on the use of genetically modified crops expressing insecticidal transgene products has the potential to drastically reduce broad-spectrum pesticide application, which kills not only the target pest but most of its natural enemies (Chrispeels & Sadava 2003). Insect-resistant transgenic crops expressing *Bacillus thuringiensis* (*Bt*) δ-endotoxins have been grown commercially since 1996 (James & Krattiger 1996) with minimal impact on nontarget organisms (Hellmich *et al*. 2001; Dutton *et al*. 2002). Their introduction is estimated to have reduced global insecticide usage (for all crops) by 14% (Phipps & Park 2002). However, because field durability of *Bt* expressing crops is predicted to be limited by the development of pest resistance (Tabashnik *et al*. 2000), other strategies are currently being investigated.

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The role of protease inhibitors (PIs) in plant defence against phytophagous pests and pathogens is now well-established. The effect of the inhibitor, not only through a direct effect on digestive proteases, but also through induction of over-synthesis of digestive enzymes in an attempt to utilize ingested food, is nutritional limitation and a consequent reduction in insect growth and development (Gatehouse *et al*. 2000). The generation of transgenic plants expressing foreign PIs is a strategy that continues to be investigated for pest control (Hilder *et al*. 1987; Mohan Babu *et al*. 2003). The mustard trypsin inhibitor-2 (MTI-2) isolated from *Sinapis alba* by Menegatti *et al*. (1992), has been demonstrated to be effective against several economically important lepidopteran insects, including the diamondback moth (*Plutella xylostella*), resulting in high mortality and significantly delayed larval development when expressed in transgenic oilseed rape (De Leo *et al*. 2001). If this approach is to be compatible with sustainable agricultural practices, then conservation of beneficial organisms in the agro-ecosystem must be a priority. Unlike *Bt* toxins which usually result in high levels of pest mortality, but are highly specific, protease inhibitors are not acutely toxic, and have a broad spectrum of activity. Over-expression of PIs in transgenic plants can thus result in beneficial insects being exposed to the insecticidal protein, via predation of surviving herbivorous insects.

Carabids (ground beetles) are abundant and important natural enemies of agricultural pests, including various lepidoptera (Lövei & Sunderland 1996). Because carabids are good indicators of ecological change, including those arising as a consequence of growing transgenic crops (Volkmar *et al*. 2002), they have the potential to serve as sensitive biodiversity indicators and are thus of high priority in risk assessment studies. They are opportunistic feeders and their major beneficial effect is maintaining pest populations at constant low levels from year to year (Finch & Collier 2000). The carabid beetle *Pterostichus madidus* (Coleoptera: Carabidae) is one of the most common and widespread of British ground beetles, occurring in woodland, grassland, and agricultural landscapes (Luff 1973).

Laboratory studies conducted to date to investigate the potential impact of PIs on carabids have been limited to the use of artificial diets to deliver the insecticidal protein to the prey (Jørgensen & Lövei 1999; Burgess *et al*. 2002). Thus far, no studies have been conducted examining the effect of PIs expressed in transgenic plants on carabid beetle fecundity and hence potential long-term impact. The aims of this paper were to evaluate the prey-mediated effects of MTI-2 expressed in transgenic oilseed rape on the carabid *P. madidus*, in terms of short-term survival and more importantly, subsequent fecundity, as well as in temporal protease regulation, under conditions where the predator had unlimited access to prey (and hence the ability to compensate by increasing prey consumption), and where intermittent feeding, as observed in the field (Cheeseman & Gillott 1987), was imposed.

Materials and methods

Reagents

The EnzChek Protease Assay Kit (Molecular Probes) was obtained from Cambridge BioScience. The enzyme substrates Z-Phe-Arg-pNA, SAAPFpNA (succinyl-ala-ala-pro-phe-p-nitroanilide) and SAAApNA (succinyl-ala-ala-ala-p-nitroanilide) and the inhibitors transepoxysuccinyl-L-leucylamido(4-guanidio)butane (E-64), phenylmethylsulphonyl fluoride (PMSF), pepstatin A, ethylenediaminetetra-acetic acid disodium salt (EDTA), L–1 — chloro-3-[4-tosylamido]-7-amino-2-heptanone.HCl, tosyl lysyl chloromethyl ketone, Tos-Lys-CH_2Cl (TLCK), L–1 — chloro-3-[4-tosylamido]-4-phenyl-2-butanone, tosyl phenylalanyl chloromethyl ketone, Tos-Phe-CH_2Cl (TPCK) and papain were purchased from Bachem (UK) Ltd. Recombinant MTI-2 (rMTI-2) was purified from *Pichia pastoris* as previously described (Volpicella *et al*. 2000), whilst cowpea trypsin inhibitor (CpTI) was affinity purified from source material (Gatehouse *et al*. 1980). Soya bean Kunitz trypsin inhibitor (SKTI) and other chemicals were from Sigma and were of analytical grade unless otherwise stated.

Plant material

Segregating (T1) transgenic spring oilseed rape seed (*Brassica napus* (L.) cv. Drakkar, line 7–36 A) expressing the serine protease inhibitor mustard trypsin inhibitor-2 was collected from primary transformants generated as previously described (De Leo *et al*. 1998). The nontransformed line Drakkar was used as the control. All plant lines were grown simultaneously under the same controlled environmental conditions: 16 h day/8 h night at a temperature of 20 °C ± 2 °C and putative transgenic plants sampled (leaves) at 8 weeks for expression of the protein; those that failed to cause a minimum of 30% inhibition of bovine trypsin activity (assayed as described below) were considered to be homozygous negative for the transgene and were not used in subsequent bioassays.

Insects

Stocks of diamondback moth (*Plutella xylostella*) from continuous culture over many generations were reared on Chinese cabbage plants in controlled environment rooms at 20 °C ± 2 °C, under a L16:D8 light regime. *Pterostichus madidus* adults were collected within the grounds of the University of Newcastle upon Tyne field station in May and June 2002. Adult beetles were maintained individually under controlled conditions (12 °C ± 2 °C, L16:D8 light

regime) and fed with larvae of *Calliphora* sp. until August 2002 (the main reproductive period) when beetles were transferred to a soil substrate, mated, and entered into trials. The condition of the beetle mandibles was used as a coarse method to separate and remove over-wintering adults from spring emerged new adults.

Production of recombinant mustard trypsin inhibitor-2 (rMTI-2)

rMTI-2 was produced and purified essentially as described by Volpicella *et al*. (2000) using *P. pastoris* (expression construct in the secretion plasmid pPIC9) as an expression system. The recombinant protein was purified from the cell culture supernatant on a HiTrap SP column (Pharmacia) with a 0–1 M NaCl linear gradient. The elutant was monitored at 280 nm and putative positive fractions assayed for inhibitory activity towards bovine trypsin (see below). The pure recombinant mustard trypsin inhibitor (rMTI-2) was dialysed exhaustively against dH_2O and finally lyophilized; biological activity was assayed by inhibition of proteolysis by tryspin on gelatin/PAGE and the identity of the purified protein was confirmed by SDS-PAGE.

Pterostichus madidus *gut enzyme preparations and protease assays*

Crude gut enzyme preparations. Adult *P. madidus* were cold anaesthetized and whole guts dissected into chilled 1 mM dithiothrietol (DTT; 1 gut/50 μL). Guts were homogenized on ice, centrifuged at 14 000 g for 5 min at 4 °C, and the supernatants were flash frozen and stored at –20 °C for future assays.

General proteolysis. General proteolytic activity was determined using the fluorescent protein substrate BODIPY-FL Casein. Briefly, gut extracts (diluted as necessary) were incubated in 50 mM buffer (as appropriate) at 25 °C in a total volume of 190 μL, and the reaction was initiated by the addition of 10 μL 10 mM substrate to give a final substrate concentration of 0.5 mM. Fluorescence was monitored in a fluorescence microtitre plate reader at 25 °C, excitation/emission maxima 485/538 nm, every 2 min over a 60-min period against appropriate controls; assays were performed in triplicate. In order to determine the pH optimum, a range of overlapping buffer systems was used: McIlvaines citric-acid phosphate (pH 4.0–7.0), *bis*-Tris propane (pH 6.5–8.0), borate-NaOH (pH 7.0–10.0) and CAPS (3-[cyclohexylamino]-1-propanesulphonic acid) (pH 10.0–11.0).

Enzyme activity in gut extracts was partially characterized using class-specific protease inhibitors. Enzyme inhibition assays were carried out at pH 8.0 (physiological gut pH as determined with a range of pH indicator papers) using borate-NaOH buffer as above. Inhibitors were pre-incubated with enzyme at 25 °C for 10 min, prior to addition of the substrate.

Inhibition assays using synthetic substrates. Assays using the specific synthetic substrates Z-Phe-Arg-pNA, SAAPFpNA and SAAApNA (final assay concentration, 0.5 mM) were carried out by incubating gut extract (diluted as necessary) with 50 mM borate-NaOH buffer (pH 8.0, physiological gut pH) in a total volume of 250 μL; inhibitors were preincubated with gut extract at 25 °C for 10 min, prior to addition of the substrate. All enzyme assays were performed in triplicate.

Determining protease activity by gel electrophoresis. Carabid gut proteolysis was qualitatively assayed using gelatin/PAGE. Gut extract (10 μg total soluble protein) was run on a 12.5% SDS-PAGE minigel, copolymerized with 0.1% gelatin; sample-loading buffer did not contain β-mercaptoethanol and samples were not boiled prior to loading. SDS was eluted from the gel following electrophoresis by incubation with 1% (v/v) Triton X-100 for 30 min at 4 °C. The gel was cut into lanes and incubated in either 50 mM borate-NaOH, pH 8.0 or 50 mM borate-NaOH, pH 8.0 with the addition of rMTI-2 to give a final concentration of 2 μM, for 3 h at 37 °C prior to staining in Kenacid blue.

Determination of transgene expression levels in oilseed rope (OSR)

Leaf samples were taken at random from both transgenic and non transformed control plants, frozen in liquid nitrogen, ground to a fine powder and extracted in 50 mM Tris-HCl buffer, pH 7.5 as previously described (Gatehouse *et al*. 1997). Extracts were centrifuged at 10 000 g for 15 min and total soluble protein of the supernatants was estimated by Bradford assay, with BSA as a standard (Bradford 1976).

MTI-2 expression in plant leaves was monitored using two different approaches: inhibition of bovine trypsin assays for rapid detection of expressors, and in-gel inhibition of proteolysis for qualitative identification of MTI-2. Trypsin inhibition assays were performed in a total volume of 250 μL in microtitre plates. Twenty micrograms of leaf protein extract, or rMTI-2, were diluted in 50 mM Tris-HCl, pH 7.5, and bovine trypsin was added to a concentration of 2.5 μg/mL (0.625 μg trypsin per assay). The assay was initiated by addition of 12.5 μL of 10 mM BApNA (final assay concentration 0.5 mM). Absorbance change was monitored at 405 nm and the remaining trypsin activity was expressed as a percent of the control. In-gel inhibition of proteolysis was carried out using gelatin/PAGE as described in De Leo *et al*. (1998). Briefly, leaf proteins (20 μg) were separated through 15% polyacrylamide gels containing 0.1% gelatin; following electrophoresis, gels

were incubated in a bovine-trypsin containing solution (2.5 μg/mL). Trypsin inhibitor activity in leaf tissue was visualized as undegraded blue-stained gelatin following staining with Kenacid blue.

Detection of MTI-2 in P. xylostella *gut tissues*

Whole guts from 15 final instar *P. xylostella* larvae, previously fed either transgenic MTI-2 expressing plants or nontransformed control plants for a minimum of 7 days, were dissected into 50 mM Tris-HCl, pH 7.5 (1 gut μL^{-1}). Following homogenization, samples were extracted for 1 h at 4 °C, and centrifuged at 13 000 g for 10 min at 4 °C. Total soluble protein was estimated by Bradford assay and samples were diluted in Tris-HCl, pH 7.5, to give a final protein concentration of 50 μg/μL. MTI-2 present in gut extracts was detected by both inhibition of bovine trypsin activity and in-gel inhibition of proteolysis as described above.

Bioassay of the effects of MTI-2 expressing oilseed rape plants on Pterostichus madidus *through the tritophic interaction*

Effects of MTI-2 expressing transgenic Oilseed Rape on the pest P. xylostella. Whole plant bioassays were conducted to determine the effects of MTI-2 expression on development and survival of the pest *P. xylostella*. Mated adult females were allowed to lay eggs on either MTI-2 expressing oilseed rape plants or nontransformed control oilseed rape plants. Neonate larvae from these egg batches were isolated and transferred to either transgenic or control plants (plants 6–8 weeks old) and survival and weight gain were recorded on a daily basis until pupation; larval instar duration was also monitored over this period. Eight plants were set up for each of the two treatments, with each plant infested with five neonates.

Effects of MTI-2 expressing transgenic oilseed rape on the predator P. madidus *via the pest*. Mated adults of *P. madidus* were divided into four groups of 20–30 insects each: (i) fed limited prey dosed on MTI-2 expressing plants; (ii) fed unlimited prey dosed on MTI-2 expressing plants; (iii) fed limited prey dosed on control plants; (iv) fed unlimited prey dosed on control plants. Insects were placed individually into 20-cm petri-dishes with a damp compost substrate. *Pterostichus madidus* adults in unlimited prey groups were fed daily on an equal weight *(ad libitum)* of final instar *P. xylostella* larvae raised on either MTI-2 expressing, or control, plants; uneaten pest remains were removed. *Pterostichus madidus* adults in limited prey groups were fed weekly on 50 mg of final instar *P. xylostella* larvae raised on either MTI-2 expressing, or control, plants; prey was totally consumed within 24 h. In all cases, pest larvae were dosed for 5 days on either MTI-2 or control plants, prior to feeding to carabids. Survival of *P. madidus* was monitored on a daily basis. Adult weight change (measured weekly) and prey consumption (measured via the daily weight of remaining uneaten prey only in unlimited prey groups) was recorded throughout the trial. The adult weight of *P. madidus* was measured prior to entry into the trial and beetles were assigned into groups with no significant difference in start weight ($n = 25$, control (U); $n = 30$, MTI-2 (U) and control (L); $n = 20$, MTI-2 (L)).

Effects of MTI-2 on predator fecundity were also assayed. Prior to entry into the trial, the adult beetles were sexed and assigned to mating pairs, which were left 72 h to ensure that females were mated. During the bioassay the number of eggs laid per female was recorded. Adult female fecundity was estimated by counts of number of eggs produced per individual.

Protease activity in P. madidus *larvae following ingestion of MTI-2 dosed prey*

Pterostichus madidus adults were fed on *P. xylostella* that had been reared on either transgenic oilseed rape or nontransformed control oilseed rape (see above). Beetles were sacrificed after either 24 h feeding or at termination of the trial (28 days). Gut extracts were prepared as previously described for *P. xylostella* with the exception that 50 μL of buffer was used per gut. Proteolytic activity was determined at pH 8.0, with BODIPY-FL Casein as substrate. The sensitivity of gut proteases to subsequent inhibition by rMTI-2 and class specific protease inhibitors was determined, as described previously.

Statistical analysis

All statistical analyses were performed with either MINITAB or STATVIEW software on an iMac computer. Differences between treatments were considered significant at the $P < 0.05$ level. When data was not normally distributed the nonparametric Mann–Whitney *U*-test replaced one-way ANOVA and unpaired *t*-tests in the analyses.

Results

Characterization of digestive proteases in the carabid beetle Pterostichus madidus

General proteolytic activity with a broad pH optimum centred on pH 8.5, with activity > 80% of maximum over the pH range of 7–10, was demonstrated in the guts of adult *Pterostichus madidus* using the fluorogenic protein substrate BODIPY-FL Casein (Fig. 1a). In-gel proteolysis assays after SDS-PAGE with gelatin as substrate revealed the presence of two clear bands corresponding to soluble proteolytic enzymes, as well as a smeared region of proteolysis as a

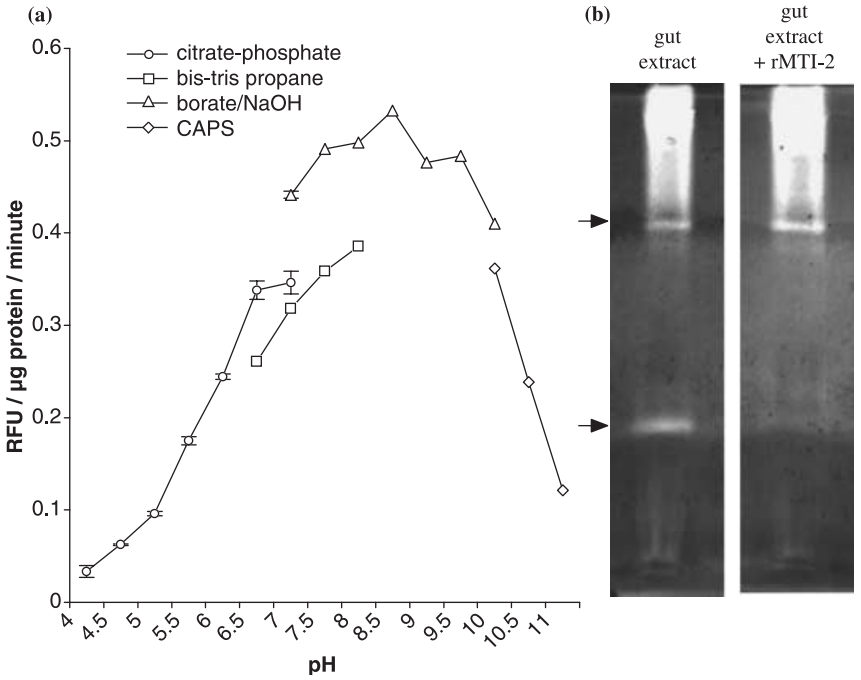

Fig. 1 (a) Determination of pH optimum of gut proteolysis in *Pterostichus madidus* using a protein substrate (BODIPY-FL Casein). Points and bars represent mean ± SE for triplicated independent determinations. (b) Visualization of protease activities present in the gut of *P. madidus* by gelatin/PAGE with and without the addition of rMTI-2. Arrows denote clear regions on the gel where gelatin has been hydrolysed.

Table 1 Inhibition of *P. madidus* gut proteolysis by class specific protease inhibitors

			Residual activity (%) at pH 8.0		
Inhibited class	Inhibitor	Final assay concentration	BODIPY-FL casein (general proteolysis)	Z-Phe-Arg-pNA (trypsin/cathepsin)	SAAPFpNA (chymotrypsin)
Cysteine	E-64	10 µM	97.1	93.3	100+
	E-64 + DTT, Brij35	10 µM	96.8	95.1	100+
Serine	PMSF	1 mM	26.6	49.6	39.6
	TLCK	100 µM	85.8	48.0	100+
	Chymostatin	100 µM	18.6	99.6	0.8
	Elastatinal	100 µM	100+	100+	100+
	MTI-2	2 µM	43.7	16.3	100+
	CpTI	1.2 µM	47.9	29.5	24.9
	SKTI	1.25 µM	27.6	30.8	13.7
	SBBI	1.2 µM	32.4	38.8	8.2
Metallo	EDTA	10 mM	93.9	100+	100+
Asparttic	PepstatinA	1 µM	89.2	100+	100+
Serine/Cysteine	Leupeptin	100 µM	94.3	5.6	93.6

result of partially soluble or aggregated enzymes (Fig. 1b, lane 1). One of the protease bands was absent when MTI-2 was included in the incubation (lane 2). This would suggest the presence of at least two distinct types of protease, one of which is inhibited by MTI-2 whereas the other is not.

Protease activity against both protein and synthetic substrates in the digestive tract of *P. madidus* was further characterized using both small molecule and protein protease inhibitors (Table 1). Only those inhibitors effective against serine proteases inhibited the hydrolysis of either protein or synthetic peptide substrates; the general cysteine protease inhibitor E-64, metallo-protease inhibitor EDTA and aspartic protease inhibitor pepstatin-A all had a relatively low effect on the hydrolysis of either labelled casein or the synthetic substrates Z-Phe-Arg-pNA and SAAPFpNA. The major digestive proteolytic activities in this species thus appear to be serine proteases. In agreement with this conclusion, the general inhibitor of serine protease activity, PMSF, inhibited hydrolysis of casein and the two synthetic protease substrates to similar extents, but the specific inhibitors chymostatin and TLCK showed different effects. Chymostatin, a specific chymotrypsin inhibitor, caused

almost complete inhibition of hydrolysis of SAAPFpNA (chymotrypsin substrate) and almost no inhibition of hydrolysis of Z-Phe-Arg-pNA (trypsin substrate), as expected, and was more effective than PMSF in inhibiting hydrolysis of casein (> 98% inhibition vs. 73% for PMSF). On the other hand, TLCK, a trypsin-specific inhibitor, had no effect on the hydrolysis of SAAPFpNA, but inhibited hydrolysis of Z-Phe-Arg-pNA by > 50%; however, it had only a marginal effect on hydrolysis of the protein substrate (14% inhibition).

The plant derived protein PIs, MTI-2, CpTI (cowpea trypsin inhibitor), SKTI (soya bean Kunitz trypsin inhibitor) and SBBI (soya bean Bowman-Birk inhibitor) all inhibited hydrolysis of the protein substrate by > 50% (56%, 52%, 72%, and 68%, respectively) when present at final concentrations in the 1–2 μM range, but showed differences in specificity when assayed for their effects on hydrolysis of the specific synthetic substrates. MTI-2 was the most effective inhibitor of Z-Phe-Arg-pNA hydrolysis (84% inhibition), but did not inhibit SAAPFpNA hydrolysis at all. CpTI, SKTI and SBBI all inhibited Z-Phe-Arg-pNA hydrolysis by 60%–70%, but were also effective inhibitors of SAAPFpNA hydrolysis (75%, 86%, and 92% inhibition, respectively). These results suggest that MTI-2 is not an effective inhibitor of the chymotrypsin-like activity present in *P. madidus* gut extracts. This is in agreement with results from in-gel proteolysis assay which demonstrated that incubation with rMTI-2 inhibited only one major band of activity (Fig. 1b).

Expression of MTI-2 in leaves of transgenic oilseed rape plants

The transgenic oilseed rape plants were segregating T1 progeny of primary transformants, and were screened to eliminate individuals homozygous negative for the transgene by assaying leaf extracts for inhibition of bovine trypsin activity. A clear distinction between plants with low levels of PI activity (as a result of endogenous PIs) and high levels of PI activity (because of transgene expression) was observed. The plants with low levels of PI activity were not used for the bioassays, and thus all the plants used were expressing MTI-2. The presence of MTI-2 in vegetative tissues of the selected transgenic plants was confirmed by inhibition of proteolysis after gelatin/PAGE (Fig. 2). After electrophoresis of leaf extracts through an acrylamide gel containing copolymerized gelatin, the gel was incubated in a trypsin solution, and then stained. Proteolysis of the gelatin gave a clear background except where hydrolysis had been inhibited, allowing the inhibitors to be visualized as stained bands. Although endogenous serine PI activity was detected as higher molecular weight bands in control oilseed rape plants (as expected), a single band with the same molecular weight as that of MTI-2 (approx. 7 kDa) was present in transgenic leaves, but absent in controls, so confirming expression of the transgene.

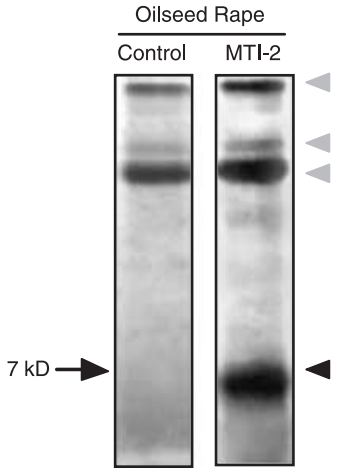

Fig. 2 Visualization of trypsin inhibitors in transgenic oilseed rape plants using gelatin/PAGE. Leaf proteins were separated through gelatin containing polyacrylamide gels (15%). The gelatin was subsequently degraded by incubation in a trypsin-containing solution. Trypsin inhibitors contained in 20 μg protein of leaf soluble protein were visualized by undegraded blue-stained gelatin located where the PI migrated. Grey arrows indicate endogenous OSR PIs, the black arrow indicates MTI-2.

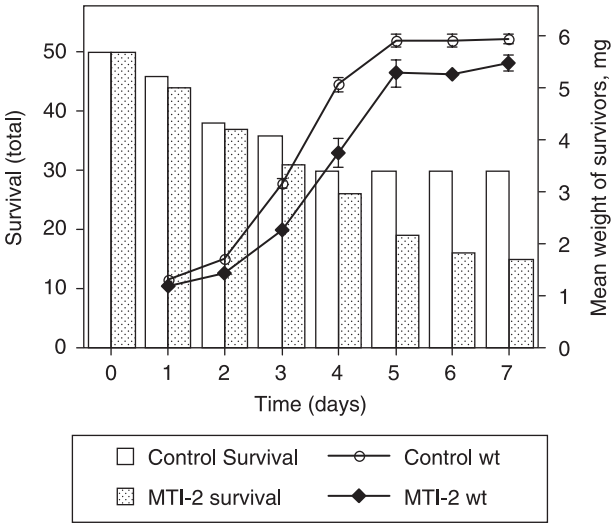

Fig. 3 Effect of MTI-2 expressed in vegetative tissues of transgenic oilseed rape on survival (bars) and growth (lines) of diamond-backed moth (*Plutella xylostella*) larvae. Bioassay was carried out over larval development from from first to final instar. Points and bars represent mean ± SE (n = 50).

Effect of MTI-2 on the pest, Plutella xylostella *and detection in gut tissue*

Plutella xylostella larvae were significantly affected by the expression of MTI-2 in oilseed rape, with a reduction in survival from 60% for neonates over 7 days on control plants to 30% on transgenic plants (Fig. 3). The decrease in

survival became significant after 5 days exposure, when larvae had been growing rapidly and must thus have been feeding on plant tissues containing the transgene product. The surviving larvae feeding on MTI-2 expressing plants achieved significantly lower mean weights compared to larvae fed on control oilseed rape foliage (*t*-test, $P = 0.019$ for final instar larvae; Fig. 3). The difference in weight between control and experimental groups of larvae was most marked during the period of most rapid growth, suggesting that the MTI-2 was exerting an antinutritional effect, leading to decreased growth and retarded development. Exposure to MTI-2 apparently led to a selection of a subgroup of larvae which were less affected by the inhibitor as the trial proceeded; the remaining larvae did not survive.

Although larvae were observed to be feeding on MTI-2 expressing rape tissues, the transgene product could be eliminated from or inactivated in the gut. The presence of functional MTI-2 in the guts of the experimental group was demonstrated by inhibition of the activity of bovine trypsin by gut extract, using BApNA as a trypsin substrate. Gut extract from control insects resulted in an increase in trypsin activity of approximately 30% resulting from endogenous trypsin-like proteases, but gut extract from insects which had fed on MTI-2 expressing plants caused an inhibition of trypsin activity (by 18%; Fig. 4a). The inhibition of bovine trypsin in this assay shows that protease inhibitor activity is present in the gut extract, and the difference between control and experimental groups of insects establishes the MTI-2 transgene product as the cause of the protease inhibition. MTI-2 was estimated to be present at levels of between 2.5 and 4.5 ng per gut in *P. xylostella* larvae on the basis of inhibiton of bovine trypsin activity. The presence of active MTI-2 in *P. xylostella* guts was further confirmed by inhibition of proteolysis after gelatin/PAGE (Fig. 4b). Proteolysis of the gelatin gave a clear background except where hydrolysis had been inhibited, allowing the inhibitors to be visualized as stained bands. Although *P. xylostella* gut proteases were observed to strongly digest gelatin (as expected), a single band with the same molecular weight as that of MTI-2 (*c.* 7 kDa) was present in MTI-2 fed group, but absent in controls, so confirming the presence of MTI-2 and hence availability to the next trophic level.

Effects of MTI-2 delivered via the herbivore Plutella xylostella *on the carabid beetle predator* Pterostichus madidus

A large-scale trial was carried out to investigate the effects of transgenic oilseed rape expressing mustard trypsin inhibitor (MTI-2) on *P. madidus* adults when delivered via the model pest *P. xylostella*. The trial used four groups of

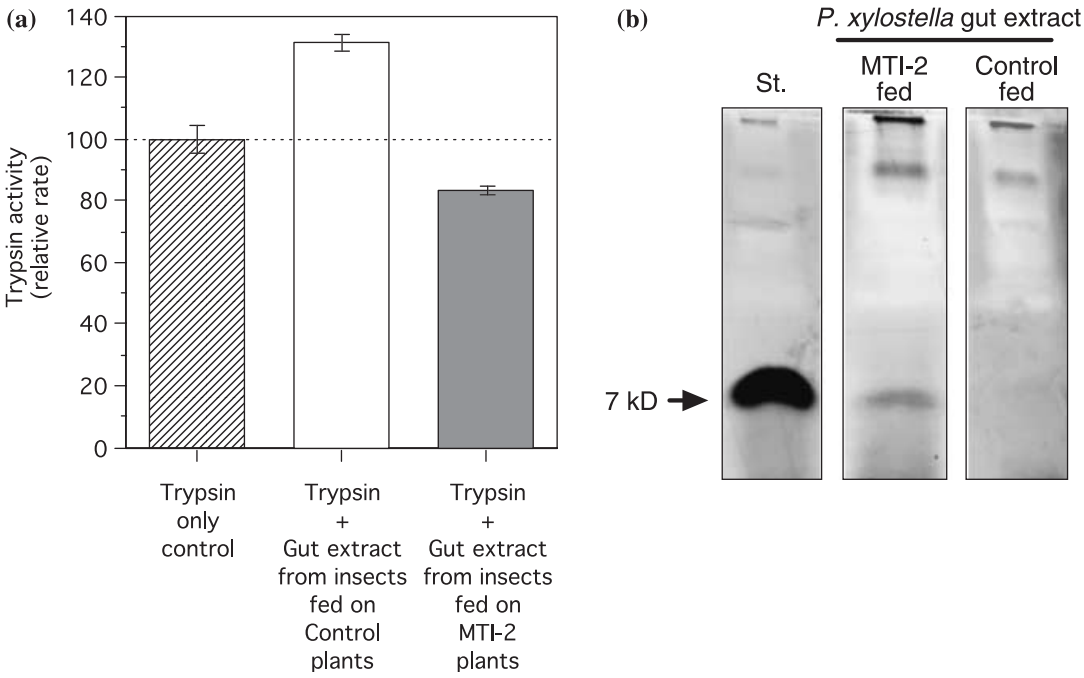

Fig. 4 (a) Detection of MTI-2 in guts of *Plutella xylostella* after feeding on MTI-2 expressing oilseed rape plants by inhibition of bovine trypsin hydrolysis of BApNA. 50 µg soluble protein was used per assay. Points and bars represent mean ± SE for triplicated independent determinations. (b) Visualization of MTI-2 in *P. xylostella* using gelatin/PAGE. Gut proteins were separated through gelatin containing polyacrylamide gels (15%). The gelatin was subsequently degraded by incubation in a trypsin-containing solution. MTI-2 contained in 50 µg of total soluble protein was visualized as undegraded blue-stained gelatin located where the PI migrated, the black arrow indicates MTI-2. St = 1 µg of rMTI-2 standard, MTI-2 fed and control-fed represent guts dissected from larvae fed the corresponding oilseed rape foliage.

insects, two groups being fed prey raised on control plants (control fed), and two groups being fed prey raised on plants expressing MTI-2 (MTI-2 fed). In addition two groups were fed prey *ad libitum* (unlimited), while the other two groups were fed controlled weights of prey on an intermittent basis (limited), to more closely model field conditions for this predator species. Each group was further divided into male and female individuals to allow for differences in feeding behaviour.

Survival in all groups was high, this being 93% and 92% for limited and unlimited control-fed beetles and 82% and 96% for limited and unlimited MTI-2 fed beetles, respectively. Statistical analyses (Kaplan–Meier Survival Analysis) were performed at four time points during the course of the 28-day trial (d7, d14, d21 and d28) and although there was a small reduction in survival of beetles fed MTI-2 when prey was limiting, this was not statistically significant at any point in time compared to any of the other treatments.

Adult beetle weight was monitored at five time points throughout the trial (0, 7, 14, 21, and 28 days). The data are presented as changes in weight over each of the four time periods 0–7 days, 7–14 days, 14–21 days, and 21–28 days in Fig. 5(a). The major factor in determining weight gain was prey supply; *P. madidus* adults experienced an overall increase in weight when fed prey in unlimited supply, irrespective of whether they had been fed control or MTI-2 fed prey; however, when prey was provided in limited supply *P. madidus* adults experienced a small overall weight loss (over the 28-day period), again irrespective of prey type.

Feeding *ad libitum* on prey containing MTI-2 led to small reductions in weight gain in *P. madidus*, although most of the differences were not statistically significant. However, after 7 days of feeding (Fig. 5a), female beetles fed unlimited prey raised on MTI-2 expressing plants had a significantly lower weight gain (approximately 50%; $P \leq 0.0001$, one-way ANOVA) when compared to the appropriate control

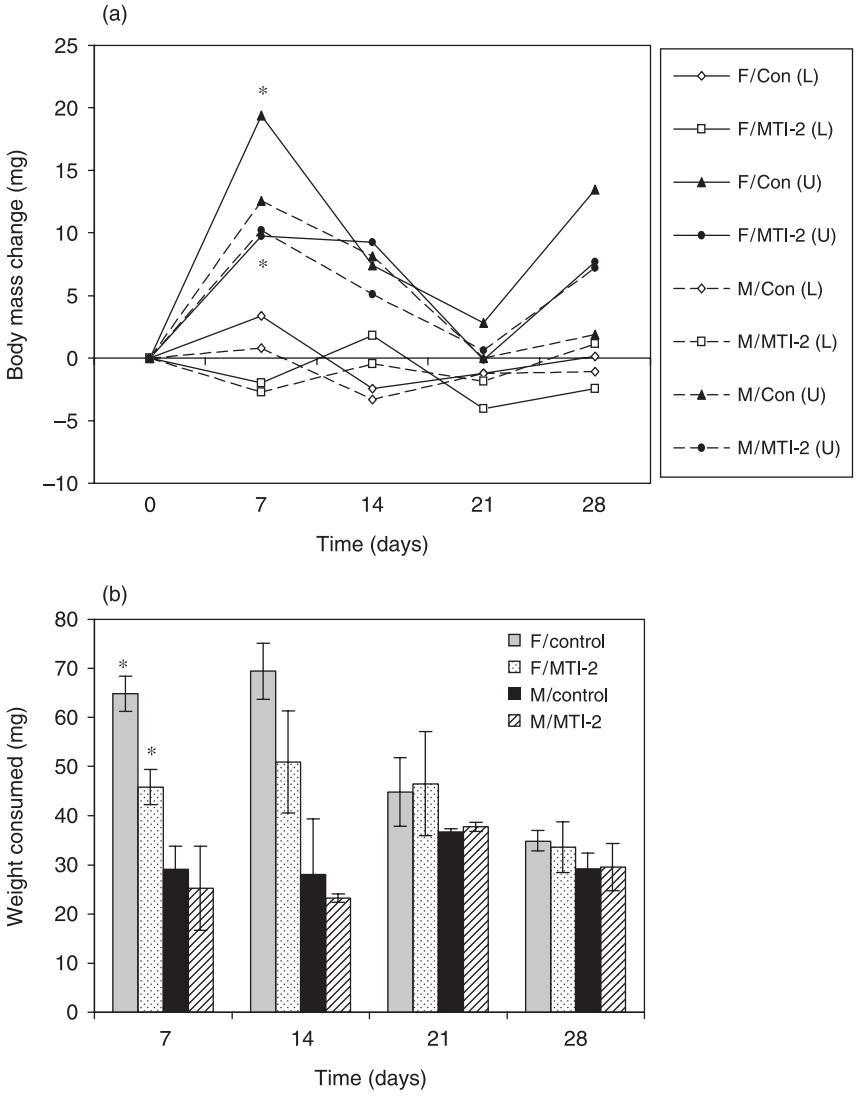

Fig. 5 (a) Mean body mass change of *Pterostichus madidus* following ingestion of MTI-2 dosed prey over successive 7-day periods of the 28-day bioassay. Prey (raised on control or MTI-2 expressing plants) was provided in either unlimited (U) or limited (L) supply. Body mass change was compared using one-way ANOVA. *Significant difference ($P < 0.05$). ($N = 25$ (cu), $N = 30$ (mu, cl) $N = 20$ (ml)). Points and bars represent mean ± SE. (b) Effect of MTI-2 on mean daily prey consumption (mg) by *P. madidus* over a 28-day period when fed prey raised on control or MTI-2 expressing plants. Weights consumed were compared using one-way ANOVA. *Significant difference ($P < 0.05$). Points and bars represent mean ± SE ($N =$ 25 and 30).

(female beetles fed unlimited prey raised on control plants). There was a corresponding significant ($P = 0.01$, one-way ANOVA) decrease in feeding at day 7 (Fig. 5b), with female beetles fed unlimited prey raised on MTI-2 expressing plants (MTI-2 unlimited group) consuming approximately 30% less MTI-2-dosed prey than controls. The differences in weight gain for this group were not present in subsequent time periods in the trial, but the female beetles fed on MTI-2 dosed prey remained smaller than those fed on prey raised on control plants. Concomitantly, there were no differences in prey consumption during the latter two weeks of the trial, although there was an overall trend for less MTI-2 prey to be consumed up to day 14 (Fig. 5b). Differences in weight gain (and prey consumption) by male beetles fed unlimited prey were not significant over any time period in the trial, and the final weights of male beetles fed MTI-2 dosed and control prey were similar to each other, and to female beetles fed on MTI-2 dosed prey. No significant differences in weight loss/gain, final weights or prey consumption were observed between any groups fed limited amounts of prey, although there was an overall trend for female beetles fed prey raised on control plants to lose less weight than any of the other groups.

The effect of feeding prey dosed with MTI-2 on the fecundity of *P. madidus* was also assayed. Adult *P. madidus* were mated prior to introduction into the trials and egg production was monitored throughout the 28-day period of the bioassay. The number of eggs laid per female was used as a measure of relative fecundity. The results showed that the presence of MTI-2 in the prey had no significant effect on mean cumulative fecundity but that availability of prey had a highly significant effect, with females fed limited prey laying only approximately 40% as many eggs as those allowed access to unlimited prey (Mann–Whitney *U*-test, $P < 0.05$, $n = 20–30$). Females fed unlimited prey dosed with MTI-2 laid approximately 6% more eggs per female than controls, whereas females fed limited prey dosed with MTI-2 laid 28% fewer eggs; neither of these differences was significant (Mann–Whitney *U*-test, $P > 0.4$, $n = 20–30$).

Effects of MTI-2 on endogenous gut proteolytic activity in Pterostichus madidus

The effects of MTI-2 on the endogenous gut proteolytic activity of *P. madidus* adults following ingestion of MTI-2 dosed prey (in limited or unlimited amounts) over time was investigated. Proteolytic activity in the gut extracts was not significantly different between groups of insects fed on unlimited and limited prey, but was generally slightly higher in female insects compared to males. Beetles that had ingested prey raised on MTI-2 expressing plants for 24 h showed significantly lower levels of hydrolytic activity against a protein substrate when compared to insects fed prey raised on control plants (Fig. 6). In the case

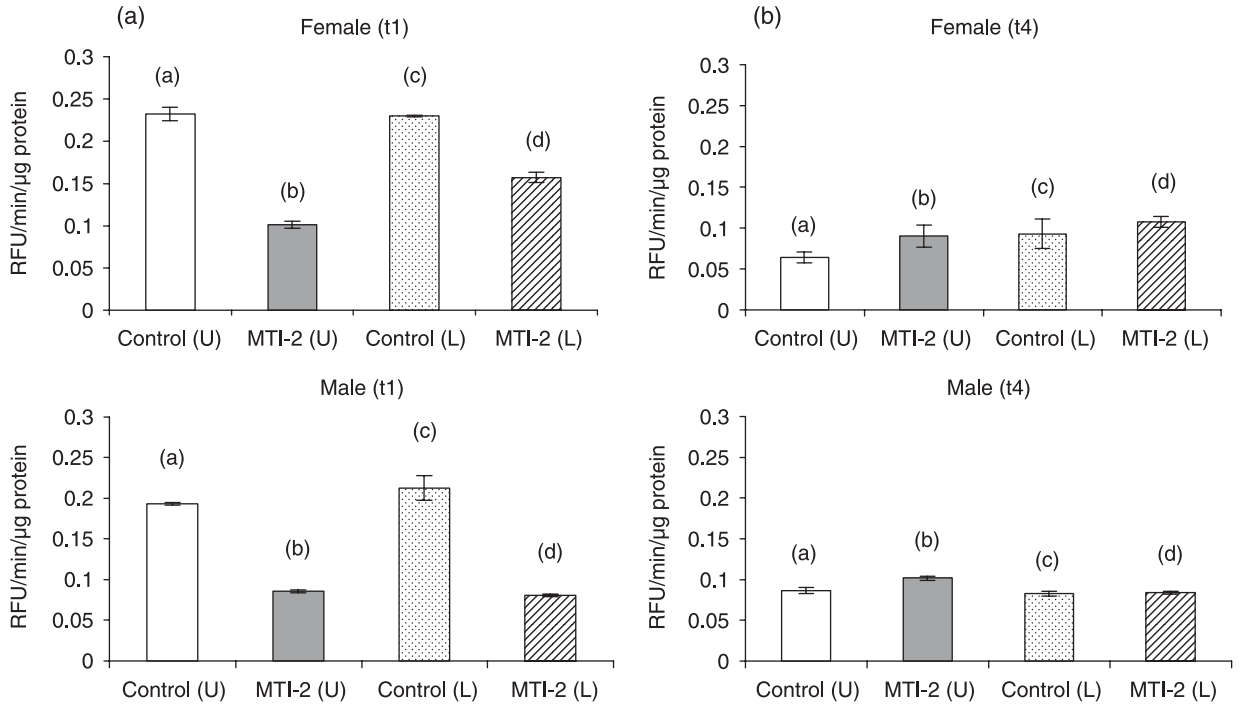

Fig. 6 Response of endogenous gut proteolytic activity in *Pterostichus madidus* to ingestion of MTI-2 dosed prey. Prey supply was either unlimited (U) or limited (L). Activities were measured at two time points: 24 h feeding (t1) and 28 days (t4). Protease activities were measured with BODIPY-FL Casein as substrate at pH 8.5. Protease activity was compared using the Students *t*-test. Different letters indicate significant difference ($P < 0.05$) for comparisons within the (U) and (L) groups. Points and bars represent mean ± SE for triplicated independent determinations.

of females, the reduction in residual activity was approximately 48% (*t*-test, $P = 0.004$) and 68% (*t*-test, $P = 0.02$) for unlimited and limited prey, respectively (Fig. 6a; t1), whilst for males the reduction in activity was approximately 44% (*t*-test, $P = 0.0002$) and 38% *t*-test, $P = 0.05$) for unlimited and limited prey, respectively. The reduction in proteolysis is most readily explained as the inhibitory effect of MTI-2 on endogenous proteases, as observed in assays *in vitro*. In contrast to the acute effects of MTI-2 on gut proteolysis, by the end of the 28-day trial (t4), no reduction in protease activity was apparent in insects fed on prey reared on MTI-2 expressing plants when compared to groups fed on control plants. This was the case for either females or males fed limited or unlimited prey (Fig. 6b). Indeed, there was a significant increase in general proteolytic activity in the groups fed prey raised on MTI-2 plants, with a 29% (*t*-test, $P = 0.018$) and 15% (*t*-test, $P = 0.03$) increase in female beetle proteolytic activity for unlimited and limited prey-fed groups, respectively, and 15% (*t*-test, $P = 0.017$) and 2% (*t*-test, $P = 0.014$) increase in male proteolytic activity for unlimited and limited prey-fed groups, respectively.

Assays of proteolytic activity in *P. madidus* gut extracts in the presence of the specific small molecule inhibitor chymostatin showed that the proteolytic enzyme profile in the beetles fed prey dosed with MTI-2 had changed in response to ingestion of the inhibitor over the duration of the feeding trial (Table 2). The main effect was to increase the level of chymotrypsin-like activity as a proportion of total proteolytic activity, shown by an increase in sensitivity to inhibition of general proteolysis. After 24 h of the feeding trial (t1), the percentage inhibition of gut extract proteolysis by chymostatin at fixed concentration ranged from 14%–24%, with no differences between insects fed on prey dosed with MTI-2 and the appropriate control. However, after 28 days (t4), differences in the sensitivity of gut proteases to chymostatin were apparent, with beetles fed MTI-2, irrespective of whether male or female or whether prey was limited or unlimited, showing increases in sensitivity to this inhibitor. There were significant differences in inhibition produced by chymostatin ($P < 0.05$), with percentage inhibition values of 24%–42% in extracts from beetles fed MTI-2 dosed prey compared to 16%–21% inhibition in extracts from beetles fed 'control' prey. A similar trend was also seen with increased sensitivity towards the general serine protease inhibitor PMSF (results not shown), although in this case the increase in sensitivity to inhibition was not so great. Low levels of inhibition (< 10%) in some insect groups after 28 days exposure to MTI-2 dosed prey were seen with the cysteine protease inhibitor E-64, the metalloprotease inihbitor EDTA and the elastase inhibitor elastatinal, whereas no inhibitory activity was observed with gut extracts from insects after 24 h exposure, or in insects fed 'control' prey (results not shown).

The change in the proteolytic enzyme profile in the predator carabid beetle as a result of long-term exposure to prey raised on MTI-2 expressing plants is confirmed by a change in the sensitivity of proteolytic activity in gut extracts to addition of exogenous rMTI-2 *in vitro*. Results (Table 2) were qualitatively and quantitatively similar for groups fed both unlimited and limited prey. After 24 h of the feeding trial, MTI-2 at fixed amount inhibited proteolysis in gut extracts of insects fed 'control' prey by approximately 35%, but inhibited proteolysis in gut extracts of insects fed MTI-2 dosed prey by approximately 55%. After 28 days of the feeding trial, MTI-2 at fixed amount showed the same level of inhibition of proteolysis in gut extracts of insects fed 'control' prey (approximately 35%). However, after 28 days insects fed prey raised on MTI-2 expressing plants showed decreased levels of inhibition by exogenous rMTI-2 (approximately 20% less). This result shows that following prolonged exposure proteolysis in the insects fed MTI-2 dosed prey has become less sensitive to inhibition.

Table 2 Inhibition of *Pterostichus madidus* gut proteases by specific inhibitors following ingestion of MTI-2 dosed prey

Inhibitor / treatment			Inhibition (%)			
			Female		Male	
			Unlimited	Limited	Unlimited	Limited
rMTI-2	T1*	*Control*	35	32	36	36
	(24h)	*MTI-2*	69*	53*	53*	52*
	T4†	*Control*	29	30	37	34
	(28d)	*MTI-2*	30	40	36	34
Chymostatin	T1	*Control*	24	14	20	14
	(24h)	*MTI-2*	23	16	15	18
	T4	*Control*	21	17	16	17
	(28d)	*MTI-2*	42‡	27*	30*	24*

*T1 = 24 hrs; †T4 = 28 days; ‡Denotes significant increase in inhibition at $P < 0.05$.

Discussion

Expression of serine PIs, such as the mustard trypsin inhibitor-2 (MTI-2), has been proposed as a means of controlling lepidopteran pests (Jouanin *et al*. 1998). However, PIs have a broad spectrum of activity in comparison to *Bt* toxins and thus may have different ecological implications for nontarget organisms. Characterization of the proteolytic activity in adults of the common British carabid beetle, *Pterostichus madidus*, in the present study demonstrated that this beetle utilizes serine proteases (both trypsin-like and chymotrypsin-like) for protein digestion. Although this activity was sensitive to inhibition by the synthetic serine-protease inhibitors PMSF, TLCK, TPCK and chymostatin, the greatest levels of inhibition were achieved with the plant-derived PIs: MTI-2, CpTI, SBTI and SBBI, with *in vitro* activity being inhibited by up to 50% by rMTI-2. These results are in agreement with those previously reported for the carabids *Pterostichus melanarious* (Gooding & Huang 1969), *Pheropsophus aequinoctialis* (Terra & Cristofoletti 1996) and *Nebria brevicollis* (Burgess *et al*. 2002). However, unlike *N. brevicollis*, no evidence for elastase-like or cysteine-like activity was shown under normal circumstances. Given that *P. madidus*, like other carabids, relies upon serine proteases, there is thus the potential for transgenic crops expressing inhibitors of such enzymes to have unintentional deleterious effects on these important generalist predators, either directly or via the prey–predator interaction. Recent studies have shown uptake of insecticidal proteins by herbivorous insect pests consuming transgenic plants (Head *et al*. 2001; Dutton *et al*. 2002; Ferry *et al*. 2003), thus there is a risk of secondary exposure to such entomophagous predators with several authors having reported negative effects resulting from exposure of nontarget organisms to PIs (Bell *et al*. 2001; Picard-Nizou *et al*. 1997; Pham-Delègue *et al*. 2000). Transgenic oilseed rape plants used in the present study were shown to express MTI-2, with expression levels up to 0.05% total soluble protein (results not shown). The presence of MTI-2 in *Plutella xylostella* (pest) larvae following ingestion of transgenic OSR was readily demonstrated, thus confirming that predating carabids would become exposed to the protein.

In agreement with previous reports, MTI-2 expressing oilseed rape had a deleterious effect on both survival and growth of the herbivorous lepidopteran *P xylostella.* De Leo *et al*. (2001) demonstrated that *P. xylostella* was the most sensitive of the lepidopteran larvae tested to MTI-2, resulting in high mortality and delayed larval development. The relatively low expression levels of MTI-2 in the transgenic oilseed rape produced a low level of acute toxicity towards *P. xylostella*, which, if used under field conditions, would result in MTI-2 dosed insects being available to entomophagous predators in the tritrophic interaction.

While the impact of plant PIs on susceptible nontarget predators has been previously investigated (Burgess *et al*. 2002; Bouchard *et al*. 2003a,b) few studies have considered the potential impact that the resulting dietary compensation may have on long-term fitness and the resources available for reproduction. Studies to investigate the effects of MTI-2 ingestion on carabids through the interaction with *P. xylostella* as prey showed that MTI-2 had no significant effect on survival, but could decrease weight gain and food consumption in the short term, although the effect was fairly small and restricted to female insects able to consume prey *ad libitum*. Female and male carabids have different nutritional requirements and show differing responses to food quality, with male beetles being less sensitive to prey quality. The effect was not significant when prey was limited, as would be the case in the field. After 7–14 days' exposure, the insects appeared to adapt to the presence of the protease inhibitor, and weight gain and food consumption became similar to insects fed on prey not dosed with MTI-2. Similar results showing that carabid beetles can overcome PI exposure have previously been reported for *Harpalus affinis* (Jørgensen & Lövei 1999) and *N. brevicollis* (Burgess *et al*. 2002). When exposed to aprotinin intoxicated prey (0.5% PI in artificial diet) *ad libitum*, *N. brevicollis* showed no differences in overall beetle survival, body mass change, or food consumption, although transient effects, such as those observed here, were also noted. Conversely, Bouchard *et al*. (2003a) recently reported that the predatory bug *Perillus bioculatus* was similarly able to respond to exposure to dietary PIs delivered through prey via the tritrophic interaction by modulation of digestive protease activity, however, survival, growth and development were found to be unaffected.

Results from the present study suggest that ability of *P. madidus* to overcome the deleterious effects of exposure to MTI-2 are based on modification of their gut protease activity. With prolonged exposure, the sensitivity of gut extracts from insects fed MTI-2 dosed prey to further inhibition by MTI-2 *in vitro* decreased. Concomitantly, sensitivity to the chymotrypsin-specific inhibitor chymostatin increased over the exposure period. MTI-2 is ineffective as an inhibitor of the chymotrypsin-like activity in *P. madidus*, as shown by assays of hydrolysis of SAAPFpNA (hydrolysed by chymotrypsin-like enzymes) *in vitro* by gut extracts, and thus the effects of this inhibitor on gut proteolysis appear to result in an up-regulation of this MTI-2 insensitive chymotrypsin-like activity. The in-gel proteolysis assay (Fig. 1b) shows the presence of a band of activity insensitive to inhibition by MTI-2.

Adaptation of gut proteolysis to dietary PIs in coleopteran species has been observed previously at the second trophic level where beetles are fed the inhibitor directly. Girard *et al*. (1998) showed that *P. chrysocephala* increased proteolytic activity by up to two-fold in response to dietary

OC-1, while Colorado potato beetle (*Leptinotarsa decemlineata*) larvae were able to compensate for exposure to OC-1 by overproduction of native digestive enzymes (Cloutier *et al*. 2000) or by producing novel proteases insensitive to inhibition (Bolter & Jongsma 1995). Interestingly, beetles at the third trophic level also appear to have the ability to overcome deleterious effects in this way. Like *P. madidus*, the carabid *N. brevicollis* (Burgess *et al*. 2002) altered protease profiles in response to ingestion of PI (aprotinin) dosed prey, with a reduction in trypsin and increase in chymotrypsin and elastase. In this case it was elastase not chymotrypsin that predominated, thus highlighting differences in the responses of individual species. Ladybird larvae (*H. axyridis*) were also observed to increase general proteolysis in response to ingestion of the cysteine PI oryzacystatin (OC-1), through consumption of dosed prey (Ferry *et al*. 2003); however, in this case the ratio of sensitive: insensitive proteases remained unchanged. Furthermore, Bouchard *et al*. (2003b) show that following consumption of PI (OC-1) dosed prey that OCI-sensitive protease activity was altered negatively, however, this inhibitory process was accompanied by a compensatory response in the predator, by which serine-type proteinases were synthesized *de novo*.

The more subtle effects that protease inhibitors may have on female fitness and fecundity are more important ecological parameters than their effects on short-term survival. Female nutrition is critical during oogenesis, affecting both the level and quality of egg production. There is a strong relationship between the level of food ingested and fecundity in carabids (VanDijk 1994; Wallin *et al*. 1992); food quality should affect the ability of females to utilize resources for egg production (Wallin *et al*. 1992). Results from the present trials, however, showed that MTI-2 did not significantly affect the fecundity of the carabid despite transient negative effects on females fed MTI-2 prey *ad libitum*, and despite resources being directed into production of MTI-2 insensitive digestive proteases, suggesting that females, despite being most sensitive to the PI, are able to overcome negative effects with no long-term impact. In fact the major contributing factor affecting fecundity was amount of food ingested, not presence of PI. Fecundity in carabids has been shown to be positively correlated with body size (Juliano 1985) and beetles fed limited prey (control or MTI-2) showed net weight loss at the end of the trial. This result is consistent with studies with female ladybirds (*H. axyridis*) which showed that consumption of PI did not have any significant impact on fecundity or subsequent egg viability (Ferry *et al*. 2003). In contrast, studies with Two-Spotted Stinkbug, *P. bioculatus*, demonstrated a 50% reduction in the fertility of females (Ashouri *et al*. 1998) fed on prey containing OC-1. However, the stinkbugs were exposed to levels of OC-1 unlikely to be encountered in the field (8–16 μg of OC-1 injected into prey/day).

In conclusion, while this study has demonstrated the potential for insecticidal proteins expressed at the first trophic level to have effects on nontarget beneficial arthropods at the third trophic level, more importantly it highlights the ability of generalist predators such as carabid beetles to respond to such challenges with minimal detrimental effects in the short term and no effects on subsequent fecundity.

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The authors' interests are in the field of Plant-Insect Interactions, with particular reference to the potential impact of transgenic plants on beneficial insect predators. Members of the consortium have differing and complementary expertise ranging from plant and insect molecular biology/biochemistry (AMRG, LJ, LRC, JAG, KE, NF), insect physiology (NF, AMRG), and ecology (EM, GP).
